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**Fast-growing *Larix kaempferi* suffers under nutrient imbalance caused by  
phosphorus fertilization in larch plantation soil**

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**Abstract** There are significant differences in the morphological and physiological responses of larch species with contrasting growth rates under fertilization. However, little is known about species-specific differences in responses to nutrient imbalance caused by fertilization. Therefore, in this study, the effects of nitrogen (N) and phosphorus (P) fertilization on the morphological, physiological and chloroplast ultrastructural traits of two contrasting larch species, fast-growing *Larix kaempferi* and slowly-growing *L. olgensis*, grown in larch plantation soil, were investigated during two growth seasons. It was shown that N and combined N and P (NP) fertilization increased plant photosynthesis, foliar N contents, chlorophyll contents, and dry mass accumulation and partitioning in aboveground organs in both larch species. Although P fertilization enhanced P accumulation, its presence reduced the N content in soluble proteins in the foliage of both larch species. Yet, P fertilization exhibited some differences in the two species: P fertilization increased photosynthesis, chlorophyll content and biomass accumulation of *L. olgensis*, while it decreased these parameters dramatically in *L. kaempferi*. P fertilization increased foliar N content in *L. olgensis*, while it reduced it in *L. kaempferi*. P fertilized *L. kaempferi* had more damaged chloroplast ultrastructure than *L. olgensis*. In addition, *L. kaempferi* exhibited lower acid phosphatase activities, and higher photosynthesis and biomass accumulation than *L. olgensis*, except under P fertilization. *L. kaempferi* allocated more biomass into needles, except under P fertilization, while *L. olgensis* allocated more into stems under fertilization. In conclusion, it was shown that nutrient imbalance caused by P fertilization has greater negative effects on a fast-growing species than on a slowly-

1 growing one, and the negative effects are related to differences in acclimation strategies,  
2 N partitioning to photosynthetic components, and P transportation and metabolism in  
3 the foliage.

4  
5 **Keywords** contrasting growth rates, nutrient imbalance, biomass allocation strategies,  
6 N partitioning, chloroplast ultrastructure

7  
8  
9 **Abbreviations** N, nitrogen; P, phosphorus;  $P_n$ , mass-based net photosynthesis rate;  $g_s$ ,  
10 stomatal conductance;  $C_i$ , intercellular CO<sub>2</sub> concentration; E, transpiration rate; Chla,  
11 chlorophyll a; Tchl, total chlorophyll;  $N_M$ , mass-based N content of needles;  $P_M$ , mass-  
12 based P content of needles;  $N_s$ , N content in soluble proteins; APA, acid phosphatase  
13 activity; TSS, total soluble sugars; NSC, non-structural carbohydrates.

## 1. Introduction

Larch plantations are widespread in northern Asia (Wang et al., 2006; Kim, 2008), where they play an important role both in the functioning of boreal forest ecosystems (Wang et al., 2001; Gao et al., 2018) and in timber production (Lei et al., 2007). N and P are essential macronutrients for plant growth and development, and nutrients that most frequently limit primary productivity in larch plantations (Yang and Zhu, 2015). Therefore, fertilization with N and P has been employed as an important management measure to improve timber yield and primary productivity in larch plantations (Chen, 2004; Na et al., 2007; Jia et al., 2010; Sun et al., 2011; Zhu et al., 2013). However, investigations on the effects of fertilization on larches have rarely considered interspecific differences and balance between nutrients, although it is known that larches possess diverse growth rates and respond differently to changes in the external environment (Zhao et al., 2007; Guo et al., 2016; Li et al., 2016), and the growth is influenced by relationships among nutrients (Leyton, 1956, 1957a). Yet, current knowledge of how eco-physiological traits of larches with contrasting growth rates respond to N and P fertilization and nutrient imbalance caused by fertilization is still scarce.

The different ways how plants allocate to shoots and roots are important strategies for adapting to various habitats, and they reflect the evolutionary history of plants (Westoby et al., 2002; Fortunel et al., 2012). Differential allocation to organs must occur at the

1    expense of other structures, as there are trade-offs in allocation among distinct organ  
2    functions (Pearcy et al., 2005; McCarthy and Enquist, 2007). N and P addition can  
3    enhance biomass accumulation and eventually increase the primary production of  
4    plantations (Zhang et al., 2016). Considering plantation management and timber  
5    production, improved understanding of plants' aboveground biomass allocation under  
6    N and P fertilization and of nutrient imbalances caused by fertilization may enhance  
7    our ability to predict the productivity of plantations in different environments. However,  
8    our understanding of variation in biomass allocation patterns across species and  
9    environments is insufficient (Poorter et al., 2012, 2015). For instance, how the  
10   allocation patterns and mechanisms of aboveground organs differ between larches with  
11   contrasting growth rates in response to N and P fertilization are still poorly understood.

12

13   During evolution, plants with contrasting-growth rates have developed different  
14   adaptations and life-history strategies (Reich, 2014). Plants with contrasting growth  
15   rates exhibit different eco-physiological traits and responses to N and/or P treatments  
16   (Carson et al., 2004; Miller and Hawkins, 2007; Bown et al., 2009; Li et al., 2012; Luo  
17   et al., 2013a; Gan et al., 2015; Li et al., 2015, 2016). The fast-growing species always  
18   exhibit greater sensitivity to N availability (Li et al., 2012) and N forms (Li et al., 2015),  
19   higher N and P use efficiencies and enzyme activities related to N assimilation and P  
20   mobilization (Gan et al., 2015), more plasticity in biomass allocation (Miller and  
21   Hawkins, 2007), and greater aboveground biomass allocation (Carson et al., 2004;  
22   Bown et al., 2009) under N and/or P fertilization compared to slowly-growing species.

1 However, plant growth relies not only on absolute amounts of either element but also  
2 on the balance between N and P (Güsewell, 2004; Ågren, 2008; Li et al., 2016). It has  
3 been showed that N and P fertilization could drive some ecosystems into nutrient  
4 imbalance, consequently leading to N or P deficiencies (Braun et al., 2010; Elser et al.,  
5 2010; Sardans et al., 2012; Li et al., 2016). Fast-growing species exploit relatively more  
6 resources when these are abundant, but there is a trade-off with the ability to avoid  
7 mortality under low-resource conditions (Kobe, 1999; Aerts and Chapin, 2000; Russo  
8 et al., 2005; Poorter and Bongers, 2006; Kursar et al., 2009; Wright and Zanne, 2010).  
9 Our previous study showed that fast-growing *L. kaempferi* suffered from N deficiency  
10 caused by P fertilization (Li et al., 2016), which suggested that although fast-growing  
11 species converge many traits to support fast growth (Reich, 2014), some of those  
12 advantages may be lost, in particular in stressful environments, e.g., under nutrient  
13 deficiencies (Zhang et al., 2014, 2017).

14

15 According to previous studies (Xu et al., 2008; Chen et al. 2010, 2011; Zhang et al.,  
16 2014, 2017; Chen et al. 2017), the slowly-growing species always exhibit better  
17 endurance and resistance under environmental stresses, e.g., drought, salinity, heavy  
18 metal stress and nutrient deficiency, than do the fast-growing ones. Consequently, we  
19 hypothesize that slowly-growing larch species are more resistant to N deficiency caused  
20 by P fertilization than fast-growing ones. To test this hypothesis, we exposed two  
21 widespread and important commercial timber species in northeastern China, fast-  
22 growing *L. kaempferi* and slowly-growing *L. olgensis* (Yu et al., 2007; Zhu et al., 2010),

1 to N and P fertilization during two consecutive growth seasons to investigate the  
2 following questions: (1) how do N and P fertilization trigger contrasting growth rates  
3 and aboveground biomass allocation among branches, trunk and needles in larches, and  
4 (2) how does *L. kaempferi* develop a disadvantage in growth under N deficiency caused  
5 by P fertilization.

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## 2. Materials and methods

### 2.1. Plant materials and experimental design

The study was conducted at the Qingyuan Experimental Station, Institute of Applied Ecology, Chinese Academy of Sciences (CAS), located in a mountain region in the eastern part of the Liaoning Province, Northeast China (41°51'N, 124°54'E, 500-1,100 m above sea level). The climate characteristics have been described in Zhu et al. (2007). Healthy one-year old saplings of *L. olgensis* and *L. kaempferi* were collected from a state-owned forest farm located in the Qingyuan Manchu Autonomous County Dasuhe, Liaoning Province, China. In May 2012, *L. olgensis* and *L. kaempferi* saplings were planted separately into 50-L plastic pots (one sapling per pot) with 40 Kg larch forest soil (organic C 41.78 g kg<sup>-1</sup>, total N 2.04 g kg<sup>-1</sup>, total P 1.23 g kg<sup>-1</sup>, available N 428.4 mg kg<sup>-1</sup>, and available P 25.2 mg kg<sup>-1</sup>). In May 2013, after one year of acclimation to natural conditions, when the saplings were two years old and had been sprouting and growing for about two weeks, 80 *L. olgensis* and 80 *L. kaempferi* saplings with an approximately similar crown size, height, and root collar diameter were chosen for N and P fertilization experiments. There were no differences in the initial size of the sampled seedlings; also no size difference among treatments at the beginning of the experiment. The experiment followed a completely randomized design with eight factorial combinations of two levels of species (*L. olgensis* and *L. kaempferi*), N (fertilization and control) and P (fertilization and control). Each species was subjected

1 to 4 regimes: control, N fertilization, P fertilization, and combined N and P fertilization.  
2 In every regime, nutrients, 4.6 g N and 2.4 g P each time, were supplied three times  
3 during the main growth season (from May to August) in 2013 and 2014, in total 13.8 g  
4 N and 7.2 g P per tree annually. The amounts of nutrients follow those in Sun et al.  
5 (2011) and Zhu et al. (2013). When supplied, the fertilizer was first dissolved in spring  
6 water and then applied into a 5-cm deep groove around each tree trunk. After  
7 fertilization, the grooves were filled up with soil. Grooves were dug carefully to avoid  
8 damaging the root. Each treatment included twenty saplings and was replicated four  
9 times (with five saplings in each replicate). All saplings were watered every two days  
10 to avoid the drought effect. The treatments started on the 20th of May 2013, and the  
11 plants were harvested on the 5th of September 2014.

12

## 13 2.2. Biomass measurements

14

15 At the end of the experiment, one sapling from each replicate was harvested randomly  
16 (thus, four saplings per treatment) and separated into needles, lateral branches, trunk  
17 and roots. The dry mass of all biomass samples was measured after oven-drying at 70 °C  
18 to a constant mass, i.e. dry mass of needles, lateral branches, trunk and roots. Stem dry  
19 mass (lateral branches dry mass + trunk dry mass), aboveground dry mass (needles dry  
20 mass + stem dry mass), total dry mass (aboveground dry mass + root dry mass),  
21 root/total mass ratio, needles/total mass ratio, trunk/total mass ratio, branches/total mass  
22 ratio, aboveground/total mass ratio, and stem/total mass ratio were calculated.

### 2.3. Leaf gas exchange rates and C, N and P content measurements

The upper mature needles of each sampling from each replicate were used for gas exchange and leaf chlorophyll pigment measurements. The net photosynthesis rate ( $P_n$ ) was measured using a portable photosynthesis measuring system, LI-COR 6400 (Li-Cor Inc., Lincoln, NE, USA), under the following conditions: leaf temperature of 25 °C, leaf air vapour pressure deficit of  $1.5 \pm 0.5$  kPa, photosynthetic photon flux density (PPFD) of  $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ , relative air humidity of 50%, and ambient  $\text{CO}_2$  concentration of  $380 \pm 5 \mu\text{mol mol}^{-1}$ . All selected needles were allowed to equilibrate for at least 5 min at  $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD before the measurements.

After  $P_n$  measurements, the used needles were picked off, the dry weights were determined to calculate mass-based  $P_n$ , and the dry samples were ground into fine powder and passed through a mesh (pore diameter ca. 275  $\mu\text{m}$ ) for the chemical element analysis. The mass-based N and P contents in needles from different treatments were determined by the semi-micro Kjeldahl method (Mitchell, 1998) and plasma emission spectroscopy (Hötscher and Hay, 1997), respectively. The C content was determined by the rapid dichromate oxidation technique (Nelson and Sommers, 1982), and the C content of each plant compartment and the whole tree was determined using the dry mass of each plant compartment.

#### 2.4. Chlorophyll pigment and protein content measurements

The upper mature needles (near the needles used for photosynthetic measurements) were clipped, and each needle sample was quickly immersed in liquid nitrogen and stored in liquid nitrogen until taken back to the laboratory, where they were stored at -70 °C for chlorophyll and protein measurements. Chlorophylls were extracted from needle samples of ~0.5 g with 10 ml 80% (v/v) aqueous solution of acetone (Lichtenthaler, 1987). After extraction, the samples were centrifuged and the absorbance of the solution was measured at 470, 663.6 and 646.6 nm using a Unicam UV-330 spectrometer (Unicam, Cambridge, UK) to determine chlorophyll a (Chla) and chlorophyll b (Chlb) according to the equations of Porra et al. (1989). Then, the total amount of chlorophyll pigments (Tchl) was calculated.

The contents of water-soluble protein fractions were determined, as described by Takashima et al. (2004). About 2 g frozen needle material was powdered in liquid nitrogen in a mortar with a pestle and homogenized in 10 ml of 100 mM sodium phosphate buffer (pH 7.5) with 0.4 M sorbitol, 2 mM MgCl<sub>2</sub>, 10 mM NaCl, 5 mM iodoacetate, 1% (v/v) polyvinylpyrrolidone, 5 mM phenylmethylsulfonyl fluoride and 5 mM dithiothreitol. The homogenate was centrifuged at 15,000 g for 30 min and the supernatant was retained for the determination of the water-soluble protein content. Water-soluble proteins were precipitated with 10% trichloroacetic acid (TCA) and washed three times with ethanol. The protein content was determined with the

1    ninhydrin method (McGrath, 1972) using bovine serum albumin as a standard. The  
2    nitrogen content of the soluble protein fraction was calculated using a conversion  
3    coefficient of 0.16 g N g<sup>-1</sup> proteins (Hikosaka and Terashima, 1996).

4

#### 5    *2.5. Acid phosphatase activity assays*

6

7    Acid phosphatase activity was assayed according to Kenton et al. (1999) with some  
8    modifications. Briefly, 0.3 g of needles were homogenized with 2.5 ml of ice-cold 50  
9    mM acetic acid–sodium acetate solution (pH 5.8) containing 1 mM EDTA, 5 mM DTT  
10    and 0.2 mM PMSF. The homogenates were centrifuged at 12,000 g for 5 min. Then,  
11    100 µl of the extract was incubated with 100 µl of *p*-nitrophenyl phosphate (*p*NPP, mg  
12    ml<sup>-1</sup>). After 30 min, the assay was stopped by the addition of 200 µl of 6 M NaOH, and  
13    the absorbance was read at 405 nm using a spectrophotometer. A *p*NPP-free incubation  
14    was included as a background for each incubation, and a standard curve in the range of  
15    0–2 mg ml<sup>-1</sup> of *p*-nitrophenol was employed.

16

#### 17    *2.6. Determination of hydrolysable amino acid contents*

18

19    Powdered dry leaf material (0.1 g) was transferred into a hydrolysis tube, 10 mL of 6  
20    mol L<sup>-1</sup> hydrochloric acid was added and the solution was frozen by refrigerant for 5  
21    min. The hydrolysis tube was vacuumed and filled with purified N<sub>2</sub> repeatedly for three  
22    times. Then, the hydrolysis tube was sealed and transferred into a constant temperature

1 drier for hydrolysis at 110 °C. After 22 h hydrolysis, the tube was removed from the  
2 dryer and cooled. The hydrolysis liquid was filtered, and the hydrolysis tube was  
3 washed with deionized water several times. The collected hydrolysis liquid was  
4 transferred into a 50 mL volumetric flask and diluted with deionized water to scale.  
5 Then, 1 mL diluted hydrolysis liquid was drawn into 5 mL volumetric flask and dried  
6 with a vacuum drier at 40-50 °C. The residue was dissolved by 1 mL of sodium citrate-  
7 hydrochloric acid buffer solution (pH 2.2) for the analysis. The composition and content  
8 of amino acids were determined by a L8800 automatic amino acid analyser (Hitachi,  
9 Tokyo, Japan).

10

### 11 *2.7. Determination of starch, total soluble sugar and non-structural carbohydrate* 12 *concentrations*

13

14 Total soluble sugars, fructose and sucrose were extracted from dried needles, stems, and  
15 roots in 80% (v/v) ethanol. Total soluble sugars were detected colorimetrically at 625  
16 nm following the anthrone–sulfuric acid method (Yemm and Willis, 1954). Fructose  
17 and sucrose were detected colorimetrically at 480 nm following the modified resorcinol  
18 method (Murata et al., 1968). Then, the starch content was determined for the pellet of  
19 plant material that remained after the removal of ethanol (Zhao et al., 2011). Solutions  
20 were filtered through Whatman GF/C filters and diluted in 10-ml volumetric flasks. The  
21 concentrations of starch as glucose equivalents were determined colorimetrically. The  
22 absorption of an enzyme blank was subtracted from each sample's absorbance prior to

1 the calculation of the sugar content. The dry mass and concentrations of total soluble  
2 sugars, fructose, sucrose and starch in each organ were used to calculate their contents  
3 in organ and in the whole tree, as well as NSC of the whole tree (Jordan et al., 2013).

#### 5 *2.8. Transmission electron microscopy*

7 Small leaf sections (2 mm in length), from the middle part of a leaf avoiding the midrib,  
8 were selected for the transmission electron microscope analysis (Zhao et al., 2009). The  
9 sections were fixed in 2.5% (v/v) glutaral pentanedial in 0.2 M phosphate-buffered  
10 saline (sodium phosphate buffer, pH 7.0) for 3 h at 22 °C and post-fixed in 2% osmium  
11 tetraoxide (OsO<sub>4</sub>) for 2 h. Then, the needles were sequentially dehydrated in 30, 50, 70  
12 and 90% acetone, and embedded in Epon 812 for 2 h. Ultrathin sections (80 nm) were  
13 sliced, stained with uranyl acetate and lead citrate, and mounted on copper grids for  
14 viewing in the H-600IV TEM (Hitachi, Tokyo, Japan) at 60.0 kV accelerating voltage.

#### 16 *2.9. Statistical analyses*

18 The statistical analyses were carried out with the Statistical Package for the Social  
19 Sciences (SPSS, Chicago, IL, USA) version 19.0. Before analyses, all data were  
20 checked for the normality and homogeneity of variances and log-transformed to correct  
21 deviations from these assumptions when needed. Three-way analyses of variance  
22 (ANOVAs) were used to evaluate the effects of species, nitrogen, phosphorus and their

- 1 interactions. Individual differences among means were determined by Tukey's tests of
- 2 One-way ANOVAs at a significance level of  $P < 0.05$ .



### 3. Results

#### 3.1. Plant biomass accumulation and partitioning

All types of fertilization increased the biomass accumulation of *L. olgensis*, except for root dry mass under NP fertilization (Fig. 1 A-F). Contrarily, N and NP fertilization increased the biomass accumulation of *L. kaempferi* (Fig. 1 A-F), while P fertilization decreased branch dry mass, stem dry mass and total dry mass (Fig. 1 B, E, F). When the two species were compared, *L. kaempferi* possessed a higher biomass accumulation than did *L. olgensis* under most conditions (Fig. 1 A-F).

All types of fertilization decreased root/total mass ratio (Fig. 2 A), increased trunk/total mass ratio, branch/total mass ratio, aboveground/total mass ratio, and stem/total mass ratio of *L. olgensis*, except for trunk/total mass ratio under N fertilization (Fig. 2 C-F). However, in *L. kaempferi*, N and NP fertilization increased needles/total mass ratio (Fig. 2 B), and NP fertilization increased aboveground/total mass ratio (Fig. 2 E), while it decreased root/total mass ratio when compared to control (Fig. 2 A). In addition, although P fertilization increased trunk/total mass ratio of *L. kaempferi* (Fig. 2 C), it decreased branch/total mass ratio significantly (Fig. 2 D).

#### 3.2. Gas exchange and chlorophyll concentration parameters

1 All fertilization increased  $P_n$ , E, Chla and Tchl of both species, except for P fertilization  
 2 decreasing  $P_n$  and having no effect on E in *L. kaempferi* (Fig. 3 A, D-F). In addition, N  
 3 fertilization increased  $g_s$  in *L. kaempferi*, P and NP fertilization increased  $C_i$  in *L.*  
 4 *olgensis*, and NP fertilization decreased  $C_i$  in *L. kaempferi* (Fig. 3 B, C). *L. kaempferi*  
 5 exhibited higher  $P_n$ , Chla and Tchl under all fertilizations (Fig. 3 A, E, F), and higher  
 6  $g_s$ ,  $C_i$  and E under N fertilization than did *L. olgensis* (Fig. 3 B-D).

7

### 8 3.3. Foliar APA, nitrogen, phosphorus and hydrolysable amino acid contents

9

10 N and NP fertilization enhanced mass-based foliar nitrogen contents ( $N_M$ ) in both  
 11 species, but P fertilization reduced  $N_M$  of *L. kaempferi* while it enhanced  $N_M$  of *L.*  
 12 *olgensis*. *L. olgensis* exhibited higher  $N_M$  than *L. kaempferi* under N and P fertilizations  
 13 (Fig. 4 A). Also, P fertilization increased mass-based foliar phosphorus contents ( $P_M$ )  
 14 (Fig. 4 B) but decreased the content of N in soluble proteins ( $N_s$ ) (Fig. 4 C) in both  
 15 species, while NP fertilization decreased  $P_M$  of *L. olgensis* (Fig. 4 B) but increased  $N_s$   
 16 of *L. kaempferi* (Fig. 4 C). In addition, N fertilization decreased APA in both species,  
 17 while P and NP fertilization increased APA in *L. olgensis*. Under all treatments, *L.*  
 18 *olgensis* showed higher APA than did *L. kaempferi* (Fig. 4 D).

19

20 N and NP fertilization generally increased the contents of hydrolysable amino acids and  
 21 TAA (Tab. 1). Exceptions were the following: arginine under N and NP fertilization,  
 22 valine, isoleucine, tyrosine, histidine, lysine and arginine under NP fertilization in *L.*

1 *olgensis*, and threonine, serine, tyrosine, histidine and proline under N fertilization in *L.*  
2 *kaempferi* (Tab. 1). P fertilization only increased lysine in *L. olgensis* and decreased  
3 serine in *L. kaempferi* (Tab. 1). In addition, most individual hydrolysable amino acid  
4 and TAA contents in *L. olgensis* were higher under N or P fertilization, but lower under  
5 NP fertilization than in *L. kaempferi* (Tab. 1).

6

### 7 3.4. Differences in sugar and C contents

8

9 All fertilizations decreased starch concentration in roots and branches, total soluble  
10 sugar (TSS) concentration in needles, branches and trunk, and NSC concentration in all  
11 organs in both species, except for starch and NSC concentrations in roots of *L.*  
12 *kaempferi* under P fertilization, TSS concentration in needles of *L. kaempferi* and NSC  
13 concentration in roots of *L. olgensis* under N fertilization (Fig. 5 A, C, F-L). N  
14 fertilization decreased needle and trunk starch concentrations and root TSS  
15 concentration in *L. kaempferi* (Fig. 5 B, D, E), and increased root TSS concentration in  
16 *L. olgensis* (Fig. 5 E). P fertilization increased trunk starch concentration in *L. kaempferi*  
17 (Fig. 5 D), and NP fertilization increased needle starch concentration and root TSS  
18 concentration, while decreasing trunk starch concentration in *L. kaempferi* (Fig. 5 B, D,  
19 E).

20

21 All fertilization increased sugar, NSC and C contents in every organ and in the whole  
22 tree in *L. olgensis*, except for root C content under NP fertilization (Figs. 6, 7 A-E). N

1 fertilization increased total sucrose and fructose contents, as well as needle, branch,  
2 trunk and total C contents in *L. kaempferi* (Figs. 6 B, C; Fig. 7 A-C, E). NP fertilization  
3 increased sugar, NSC and C contents in every organ and in the whole tree in *L.*  
4 *kaempferi* (Figs. 6, 7 A-E). P fertilization decreased sugar and NSC contents in every  
5 organ and in the whole tree, and branch and total C contents in *L. kaempferi* (Fig. 6 A-  
6 E; Fig 7 B, E).

### 8 3.5. Differences in needle ultrastructure

9  
10 When compared with control conditions, both species had greater chloroplast volumes  
11 under N and NP fertilization (Fig. 5 A-D, G, H), while under P fertilization, *L. olgensis*  
12 exhibited normal and intact organelles (Fig. 5 E), but *L. kaempferi* organelles showed a  
13 strong degree of destruction with incomplete chloroplast structures, damaged thylakoid  
14 membranes, scattered laminated structures, and disordered mitochondria (Fig. 5 F).  
15 Under N and NP fertilization, both species showed smooth and continuous cell  
16 membranes and cell walls, and numerous organelles in the cytoplasm. They also  
17 possessed typical chloroplast structures and well-arranged thylakoids in the granal  
18 regions, and clear mitochondrial cristae (Fig. 5 C, D, G, H). In addition, both species  
19 exhibited, to some degree, starch and plastoglobuli accumulation in the cytoplasm  
20 under each treatment (Fig. 5 A-H).

## 4. Discussion

### 4.1. Changes in biomass accumulation and partitioning under N and P fertilization

N and P fertilizations during two growing seasons influenced growth, above- and below-ground biomass allocations, as well as the physiological and biochemical parameters of *L. olgensis* and *L. kaempferi* needles. A significant effect of species was discovered, which indicated that there are species-specific eco-physiological responses to N and P fertilization. Except for P fertilization decreasing the growth of stem, branch, and whole-plant mass of *L. kaempferi*, fertilizations significantly increased the biomass accumulation of each organ and the whole plant in *L. olgensis* and *L. kaempferi*. Both species exhibited similar changes in biomass allocation alteration from below- to above-ground organs. These results are consistent with previous studies (Gedroc et al., 1996; Shipley and Meziane, 2002; Poorter et al., 2012; Freschet et al., 2013). Nevertheless, when nutrient limitation was relieved by fertilizations, these two species displayed highly contrasting above-ground biomass allocation patterns among needles, trunk and branches, which have different physiological functions (Pearcy et al., 2005). Our results suggest that *L. kaempferi* prefers allocation to needles, while *L. olgensis* prefers biomass allocation to stems, as long as there are adequate nutrient supplies, except for *L. kaempferi* under P fertilization. Different above-ground biomass allocation patterns in the two species showed the presence of contrasting trade-offs between organs that provide different functions related to fertilization (Poorter and Nagel, 2000;

Yagi, 2000; Taneda and Tatenno, 2004; Poorter et al., 2012). The biomass of plantations has been of a critical importance for economic and ecological requirements (Egnell, 2011), and the pattern of biomass allocation affects plant growth, biogeochemical cycling, ecosystem function (Peri et al., 2010; Uri et al., 2014) and the amounts of timber harvest in different scenarios (e.g., stem-only harvesting and whole-tree harvesting) (Yan et al., 2017). Therefore, it is necessary to take into account species-specific responses and biomass allocation patterns to fertilization when designing plantation management.

#### *4.2. Different responses of physiological and biochemical parameters, and needle ultrastructure under P fertilization*

Photosynthetic capacity is one of the core nutrition-related physiological traits of leaves (Wright et al., 2004). Our results showing that different types of N and P fertilization increase  $P_n$  significantly are consistent with many previous studies on N and P fertilization effects (Fatichi et al., 2014; Zhang et al., 2014; Guo et al., 2016), with the exception that P fertilization reduced  $P_n$  in *L. kaempferi* (Fig. 3 A). It has been previously verified that P fertilization induces N deficiency in this species when grown in larch soil (Li et al., 2016). In the present study, the N content of soluble proteins (Ns), represented by Rubisco (Takashima et al., 2004; Feng et al., 2009; Dong et al., 2015), was measured and found to decrease in both species under P fertilization (Fig. 4 C). The amount of synthesized Rubisco is strongly controlled by nitrogen availability (Kattge et al., 2009). Also, variation in the amount of free amino acids, which represent the second most N-containing material in the leaves of woody plants (Millard, 1988; Ruan

1 et al., 2010), could reflect the influence of nutrients on N metabolism and acclimation  
2 (Fougere et al., 1991; Schubert et al., 1995). In previous reports on conifers (Påhlsson  
3 et al., 1992; Calanni et al., 1999), it has been demonstrated that when N supply is  
4 sufficient, N is often used to increase amino acids. In the present study, the  
5 accumulation of most hydrolysable amino acids was greatly enhanced under N and NP  
6 fertilization, while only little change was detected under P fertilization in both species  
7 (Tab. 1). The results of Ns and amino acids agree with previous studies showing that P  
8 fertilization induces N deficiency in the two *Larix* species (Li et al., 2016). However,  
9 the two species respond differently to N deficiency:  $P_n$  of only *L. kaempferi* reduces  
10 under N deficiency caused by P fertilization.

11

12 Generally, photosynthetic inhibition is classified to be due to stomatal and nonstomatal  
13 limitation. However, a similar tendency between  $C_i$  and  $g_s$  was observed in both species  
14 under P fertilization (Fig. 3 B, C), indicating that stomatal limitation did not contribute  
15 to the negative effect of P fertilization on the photosynthesis of *L. kaempferi* (see also  
16 Bown et al., 2009; Warren, 2011). Thus, the decrease of  $P_n$  in *L. kaempferi* is possibly  
17 due to non-stomatal factors, such as the chlorophyll content, N content and allocation,  
18 Rubisco content and activity, as well as the structures of organelles related to  
19 photosynthesis (Zhang et al., 2014). Nevertheless, P fertilization increased Chla and  
20 Tchl in both species. Therefore, chlorophyll contents did not cause decreases in  $P_n$  (Fig.  
21 3 E, F). A question arises, which factors are actually responsible for the reduction of  $P_n$   
22 in *L. kaempferi* under P fertilization? It has been reported that there is a strong positive  
23 correlation between foliar N contents and  $P_n$  (Takashima et al., 2004; Wright et al., 2004;  
24 Zhang et al., 2014; Li et al., 2016), and N plays an essential role in photosynthetic  
25 processes (Güsewell, 2004). This is comprehensible, because N is a vital constituent of

1 proteins, chlorophylls and other substances in plants (Luo et al., 2013b). Usually 71-  
 2 77% of the leaf nitrogen is found in the form of proteins, most of which are involved in  
 3 the photosynthetic machinery (Takashima et al., 2004; Yasumura et al., 2007). Thus,  
 4 the decrease in  $P_n$  detected in *L. kaempferi* in the present study was induced to some  
 5 degree by constrained  $N_M$  under P fertilization (Fig. 3 A), as previously proposed by  
 6 Reich et al. (1994). Decreased foliar N concentrations have been detected under P  
 7 fertilization also in *L. kaempferi* (*L. leptolepis* in van Goor, 1953), *Rhizophora mangle*  
 8 (Lovelock et al., 2004), four dominant savannah woody species (Bucci et al., 2006) and  
 9 larch hybrid seedlings (Fujita et al., 2018). However, the reason for this phenomenon is  
 10 not clear, but it may be caused by the response of water relations and hydraulic  
 11 architecture to N or P addition (Goldstein et al., 2013). In a previous study, it has been  
 12 revealed that the optimum N and P concentrations in *L. kaempferi* are 2.8 and 0.4  
 13 percent dry mass (Leyton, 1957a). In this study, the needle N and P concentrations  
 14 ranged from 8.8 to 13.6 mg g<sup>-1</sup> and from 1.9 to 5.2 mg g<sup>-1</sup>, respectively (Fig. 4 A, B).  
 15 The N values were generally higher than the N concentrations reported in Guo et al.  
 16 (2016) but lower than those in Yan et al. (2017), and the amounts of P were higher than  
 17 P concentrations in Wang et al. (2018). However, the mean N concentration (11.2 mg  
 18 g<sup>-1</sup>) observed in our study was more or less the same as detected previously in conifer  
 19 and gymnosperm leaves (11.7 mg g<sup>-1</sup>) (Han et al., 2005). The reason for the discrepancy  
 20 between our study and others is probably attributed to growth conditions (e.g., soil  
 21 background, stand age, species relationships) and experimental methods. After all, even  
 22 in the same tree, the composition of the foliage is not constant but varies according to  
 23 its location on the crown, its age (i.e., in conifers bearing more than one year's needles),  
 24 and the time of the year (Leyton, 1957b).

25



1 The presence of disrupted needle ultrastructure suggests that N deficiency caused by P  
2 fertilization exhibited more negative effects on *L. kaempferi*. These results are  
3 consistent with a previous investigation conducted on fast-growing *P. cathayana*  
4 females under N deficiency (Zhang et al., 2014). It has been reported that there is an  
5 excessive P uptake under a low N supply, but a dilution in the P concentration under a  
6 high N supply (Lajtha and Klein, 1988). In addition, symbiosis with ectomycorrhizal  
7 (EMC) fungi is prevalent in larch species under nutrient deficiency; larch seedlings  
8 infected with ECM have an enhanced N and P uptake (Kayama et al., 2015). Similarly,  
9 as in our study, P fertilization has been previously found to suppress belowground,  
10 aboveground and total dry mass in three larch species when taken EMC infection into  
11 account under N and P fertilization (Wang et al. 2018), and variation has been observed  
12 in needle P concentrations in Dahurian larch and Japanese larch under N and P  
13 fertilization (Wang et al. 2018). These results indicate that although symbiosis with  
14 EMC fungi plays an important role in leaf nutrient concentrations under deficient  
15 conditions, the effect may become weaker under nutrient addition. Also, excessive P  
16 accumulation may cause toxic effects (Shane et al., 2004). The physiology of P toxicity  
17 is not well understood. P toxicity symptoms (e.g., growth inhibition and photosynthetic  
18 rate suppression) have been thought to result from the interaction of cellular P with  
19 micronutrients (e.g. Zn, Cu, Mn and Fe), or from interference with leaf water relations  
20 at high cellular P (Shane et al., 2004). In the future, there is a need to explore the  
21 physiological mechanism of P toxicity in *L. kaempferi* under P fertilization. On the other  
22 hand, we found that *L. kaempferi* had a lower APA level than did *L. olgensis* under all  
23 treatments (Fig. 4 D). APA plays an important role in the production, transport and  
24 recycling of phosphates (Duff et al., 1991; Zhang et al., 2014). Such results indicate  
25 that *L. kaempferi* inherently possesses a lower efficiency or lower ability of P

1 metabolism than does *L. olgensis*. Therefore, we propose that *L. kaempferi* with a lower  
2 ability of P production, transport and recycling might suffer more toxic effects as  
3 induced by P accumulation. Also, decreasing  $N_M$  in *L. kaempferi* under P fertilization  
4 could negatively impact the P metabolism (Fig 4 A), because APA requires a significant  
5 investment of N (Houlton et al., 2008).

6

7 Sugars perform important regulatory functions in the life cycle of plants, including  
8 photosynthesis (Rolland et al., 2002; Halford and Paul, 2003; Lloyd and Zakhleniuk,  
9 2004) and carbohydrate partitioning (Rook and Bevan, 2003). N and P, as essential  
10 macronutrients for plant growth and development, affect, to various extent, sugar  
11 metabolism and/or carbohydrate partitioning between source and sink tissues (Hermans  
12 et al., 2006; Simon et al., 2010). The dynamics of NSCs is considered being an indicator  
13 of the source-sink balance, such as photosynthesis vs. respiration and growth  
14 (Richardson et al., 2013). In our study, in both species, sugar and NSC concentrations  
15 of each organ generally exhibited decreasing tendencies after N and NP fertilization  
16 (Fig. 5 A-L), which is consistent with previous reports (Curtis et al., 2000; Ludovici et  
17 al., 2002). This happens mainly because fertilization increases growth rates and the  
18 production of new organs, which enhances the utilization of carbohydrates (enhanced  
19 sink strength) (Millard and Grelet 2010) and then decreases concentrations of  
20 carbohydrates in tissues (Moore et al., 1999; Villar-Salvador et al., 2013). Furthermore,  
21 although sugar and NSC concentrations of each organ in both species mostly exhibited  
22 decreasing tendencies under P fertilization, decreases in *L. olgensis* were driven by  
23 enhanced sink strength, while decreases in *L. kaempferi* were caused by an insufficient

C source supply, as proven by decreased  $P_n$ , total NSC and C content in the whole tree as well as lowered biomass accumulation under P fertilization (Fig. 3 A; Fig. 6, 7 E; Fig 1 B, E, F).

Plant growth relies on the absolute nutrient status, as well as on the balance among nutrients (Güsewell, 2004; Garrish et al., 2010). The findings of our research suggest that larches with different growth rates grown in N and P co-limited soils exhibit opposite or different intensity responses in plant growth. In addition, although low N availability might limit plants' responses to P fertilization (Fujita et al., 2010), N fertilization could benefit plants by allocating N to phosphatase production (Menge and Field, 2007; Hogan et al., 2010; Marklein and Houlton, 2012). Plants favor consuming N and adjusting C allocation to meet the demand for P (Deng et al., 2016). In accordance with those views, the results of the present study provide further evidence that N fertilization improves while P fertilization restrains the growth of *L. kaempferi*.

## 5. Conclusions

Although N and NP fertilization enhanced the growth of both studied *Larix* species, there were significant differences between *L. olgensis* and *L. kaempferi* in the responses to N deficiency caused by P fertilization when grown in larch soil: P fertilization increased growth, biomass accumulation, photosynthetic rate,  $N_M$ , NSC and C content in *L. olgensis*, while it decreased these parameters in *L. kaempferi*. Several factors are responsible for species-specific differences in N deficiency effects caused by P fertilization, such as biomass partitioning, P fertilization modulating N content and partitioning in mesophyll cells, P accumulation and P metabolism-related enzyme

1 activities, as well as ultrastructural changes in needles. The higher  $N_M$  and APA levels  
2 of the slowly-growing species *L. olgensis* enhance P metabolism that prevents the toxic  
3 effect caused by the excess P accumulation. While the fast-growing species *L. kaempferi*  
4 suffers from N deficiency and has disrupted chloroplasts, which inhibit photosynthesis,  
5 leading to decreased NSC and C contents, and, eventually, to a lower biomass  
6 accumulation and growth. Therefore, it is evident that the two species with contrasting  
7 growth rates employ different strategies to undergo N deficiency induced by P  
8 fertilization. Slowly-growing *L. olgensis* possesses a more effective N allocation in  
9 photosynthesis and P metabolism than fast-growing *L. kaempferi*. Our results were also  
10 consistent with previous studies indicating that fast-growing species are more sensitive  
11 and less tolerant to stressful external environments. Furthermore, various studies agree  
12 with the viewpoint that human activities have induced N and P imbalances and  
13 aggravated P limitation in temperate forests (Tessier and Raynal, 2003; Han et al., 2005;  
14 Xu et al., 2017; Zheng et al., 2017), including larch plantations (Yan et al., 2017).  
15 Because there are different responses among larch species to P fertilization and nutrient  
16 imbalance, the specific effects of fertilization should be considered when managing  
17 plantations for carbon binding or biomass production in future. For example, supplying  
18 balanced N and P nutrients would benefit *L. kaempferi* growth more than providing P  
19 alone when there is P limitation caused by human activities. The results generated in  
20 our study could enhance our ability to maintain the stability of larch plantations and  
21 improve their productivity, especially in Northeast China.

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2 collection, analysis and writing, Guoxi Wu and Qingxue Guo had a significant  
3 contribution to data collection and analysis, Helena Korpelainen had a significant  
4 contribution to the interpretation of data and manuscript preparation, and Chunyang Li  
5 (the corresponding author) had the overall responsibility for experimental design and  
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7

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**Table 1.** Hydrolysable amino acid contents of *L. olgensis* and *L. kaempferi* needles as affected by N and P fertilization.

Hydrolysable amino acid (mg g <sup>-1</sup> DW)	<i>L. olgensis</i>				<i>L. kaempferi</i>			
	C	N	P	NP	C	N	P	NP
Aspartic acid	10.95 cd	14.07 a	11.51 bc	12.27 b	10.19 de	11.37 bc	9.55 e	14.66 a
Threonine	5.65 cd	8.86 a	5.96 c	6.05 c	5.38 cd	5.89 c	4.96 d	7.54 b
Serine	5.34 de	7.41 a	5.48 cd	5.87 c	4.92 e	5.31 de	4.43 f	6.92 b
Glutamic acid	12.55 de	17.54 a	13.33 cd	14.06 c	11.42 f	12.69 de	11.88 ef	16.27 b
Glycine	6.56 cd	8.53 a	6.84 bc	7.26 b	6.09 de	6.76 bc	5.62 e	8.73 a
Alanine	7.65 de	11.64 a	8.16 cd	8.40 c	7.13 e	7.88 cd	7.08 e	10.22 b
Valine	7.16 bc	9.06 a	7.64 b	7.87 b	6.60 c	7.55 b	6.43 c	9.58 a
Isoleucine	5.84 cd	7.14 b	6.10 c	6.31 c	5.39 de	6.09 c	5.09 e	7.73 a
Leucine	11.05 de	15.86 a	11.63 cd	12.22 c	10.31 ef	11.57 cd	9.92 f	14.76 b
Tyrosine	4.15 cd	6.45 a	4.26 cd	4.66 c	3.95 de	4.40 cd	3.48 e	5.78 b
Phenylalanine	6.96 def	10.25 a	7.30 cde	7.79 c	6.64 ef	7.40 cd	6.30 f	9.39 b
Histidine	2.84 cd	5.54 a	2.93 bcd	3.15 bc	2.66 d	2.97 bcd	2.54 d	3.36 b
Lysine	7.26 cd	8.70 b	8.55 b	8.17 bc	6.74 d	8.13 bc	6.92 d	10.23 a
Arginine	7.55 bc	7.84 b	8.00 b	7.83 bc	7.07 cd	7.33 bcd	6.73 d	9.82 a
Proline	5.54 def	9.37 a	5.91 cd	6.16 c	5.21 ef	5.75 cde	5.17 f	7.31 b
TAA	110.62 cd	157.77 a	116.56 bc	122.39 b	103.23 de	113.85 bc	98.09 e	146.20 a

Each value is the mean ( $n = 4$ ). Values not sharing the same letters in the same row are significantly different at  $P < 0.05$  according Tukey's test. C, control; N, nitrogen fertilization; P, phosphorus fertilization; NP, combined N and P fertilization.

1 **Figure legends**

2

3 **Figure 1.** The total dry mass and its components in *L. olgensis* and *L. kaempferi* as  
4 affected by N and P fertilization. Each value is the mean  $\pm$  SE ( $n = 4$ ). A, root dry mass;  
5 B, branch dry mass; C, trunk dry mass; D, needle dry mass; E, total stem dry mass; F,  
6 total dry mass. The values not sharing the same letters are significantly different at  $P <$   
7 0.05 according to Tukey's test. C, control; N, nitrogen fertilization; P, phosphorus  
8 fertilization; NP, combined N and P fertilization.

9

10 **Figure 2.** Dry mass partitioning in *L. olgensis* and *L. kaempferi* as affected by N and P  
11 fertilization. Each value is the mean  $\pm$  SE ( $n = 4$ ). A, root/total mass ratio; B, needle/total  
12 mass ratio; C, trunk/total mass ratio; D, branch/total mass ratio; E, aboveground/total  
13 mass ratio; F, stem/total mass ratio; The values not sharing the same letters are  
14 significantly different at  $P < 0.05$  according to Tukey's test. C, control; N, nitrogen  
15 fertilization; P, phosphorus fertilization; NP, combined N and P fertilization.

16

17 **Figure 3.** Gas exchange parameters and chlorophyll contents of *L. olgensis* and *L.*  
18 *kaempferi* as affected by N and P fertilization. Each value is the mean  $\pm$  SE ( $n = 4$ ). A,  
19  $P_n$ , mass-based net photosynthetic rate; B,  $g_s$ , stomatal conductance; C,  $C_i$ , intercellular  
20  $CO_2$  concentration; D, E, transpiration rate; E, Chla, chlorophyll a content; F, Tchl, total  
21 chlorophyll content; The values not sharing the same letters are significantly different  
22 at  $P < 0.05$  according to Tukey's test. C, control; N, nitrogen fertilization; P, phosphorus

fertilization; NP, combined N and P fertilization.

**Figure 4.** (A and B) Mass-based foliage N and P content ( $N_M$ ,  $P_M$ ), (C) N content of the soluble protein fraction ( $N_s$ ), and (D) acid phosphatase activity (APA) in *L. olgensis* and *L. kaempferi* as affected by N and P fertilization. Each value is the mean  $\pm$  SE ( $n = 4$ ). The values not sharing the same letters are significantly different at  $P < 0.05$  according to Tukey's test. C, control; N, nitrogen fertilization; P, phosphorus fertilization; NP, combined N and P fertilization.

**Figure 5.** (A - D) Starch concentration in roots, leaves, branches and trunks, (E - H) total soluble sugar (TSS) concentration in roots, leaves, branches and trunks, and (I - L) non-structural carbohydrate (NSC) concentration in roots, leaves, branches and trunks in *L. olgensis* and *L. kaempferi* as affected by N and P fertilization. Each value is the mean  $\pm$  SE ( $n = 4$ ). The values not sharing the same letters are significantly different at  $P < 0.05$  according to Tukey's test. C, control; N, nitrogen fertilization; P, phosphorus fertilization; NP, combined N and P fertilization.

**Figure 6.** (A - C) Total starch, sucrose and fructose content, (D) total soluble sugar (TSS) content, and (E) total non-structural carbohydrate (NSC) content of *L. olgensis* and *L. kaempferi* as affected by N and P fertilization. Each value is the mean  $\pm$  SE ( $n = 4$ ). The values not sharing the same letters are significantly different at  $P < 0.05$  according to Tukey's test. C, control; N, nitrogen fertilization; P, phosphorus fertilization; NP,

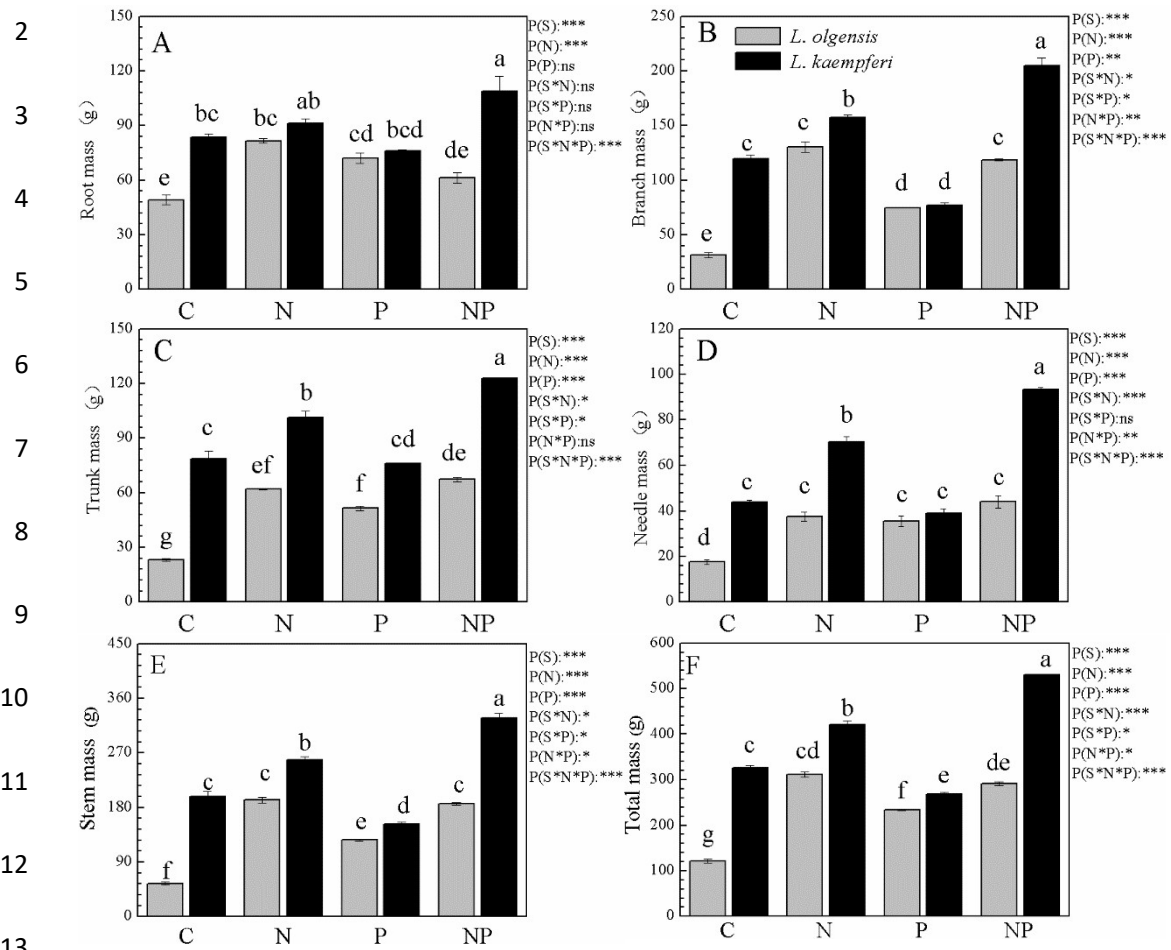


combined N and P fertilization.

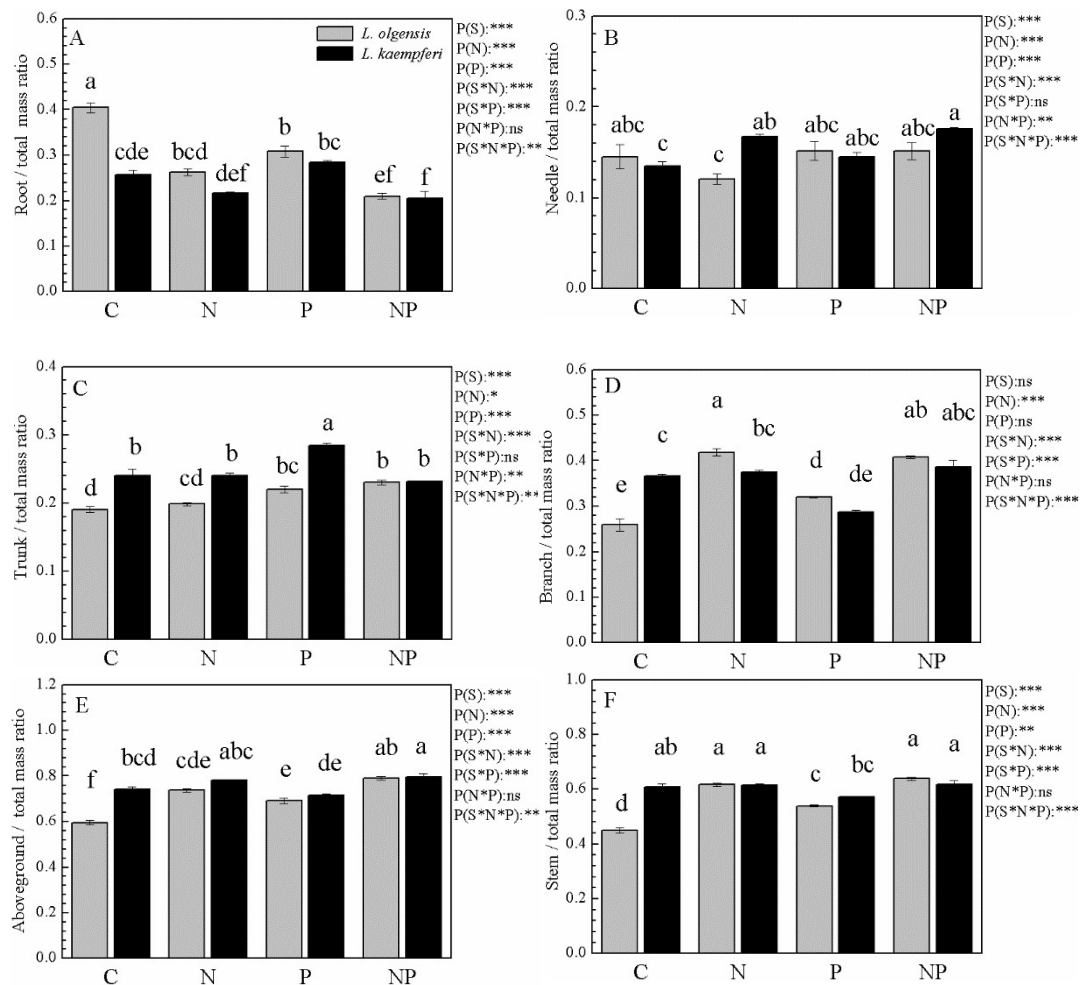
**Figure 7.** (A - D) Root, branch, trunk and needle carbon content, and (E) total carbon content of *L. olgensis* and *L. kaempferi* as affected by N and P fertilization. Each value is the mean  $\pm$  SE ( $n = 4$ ). The values not sharing the same letters are significantly different at  $P < 0.05$  according to Tukey's test. C, control; N, nitrogen fertilization; P, phosphorus fertilization; NP, combined N and P fertilization.

**Figure 8.** Transmission electron micrographs of mesophyll cells in *L. olgensis* and *L. kaempferi* as affected by N and P fertilization. A, Control *L. olgensis*; B, Control *L. kaempferi*; C, N fertilization *L. olgensis*; D, N fertilization *L. kaempferi*; E, P fertilization *L. olgensis*; F, P fertilization *L. kaempferi*; G, combined N and P fertilization *L. olgensis*; H, combined N and P fertilization *L. kaempferi*. The bars shown are 1  $\mu$ m. Ch, chloroplast; CW, cell wall; M, mitochondrion; P, plastoglobulus; SG, starch grain; V, vacuole.

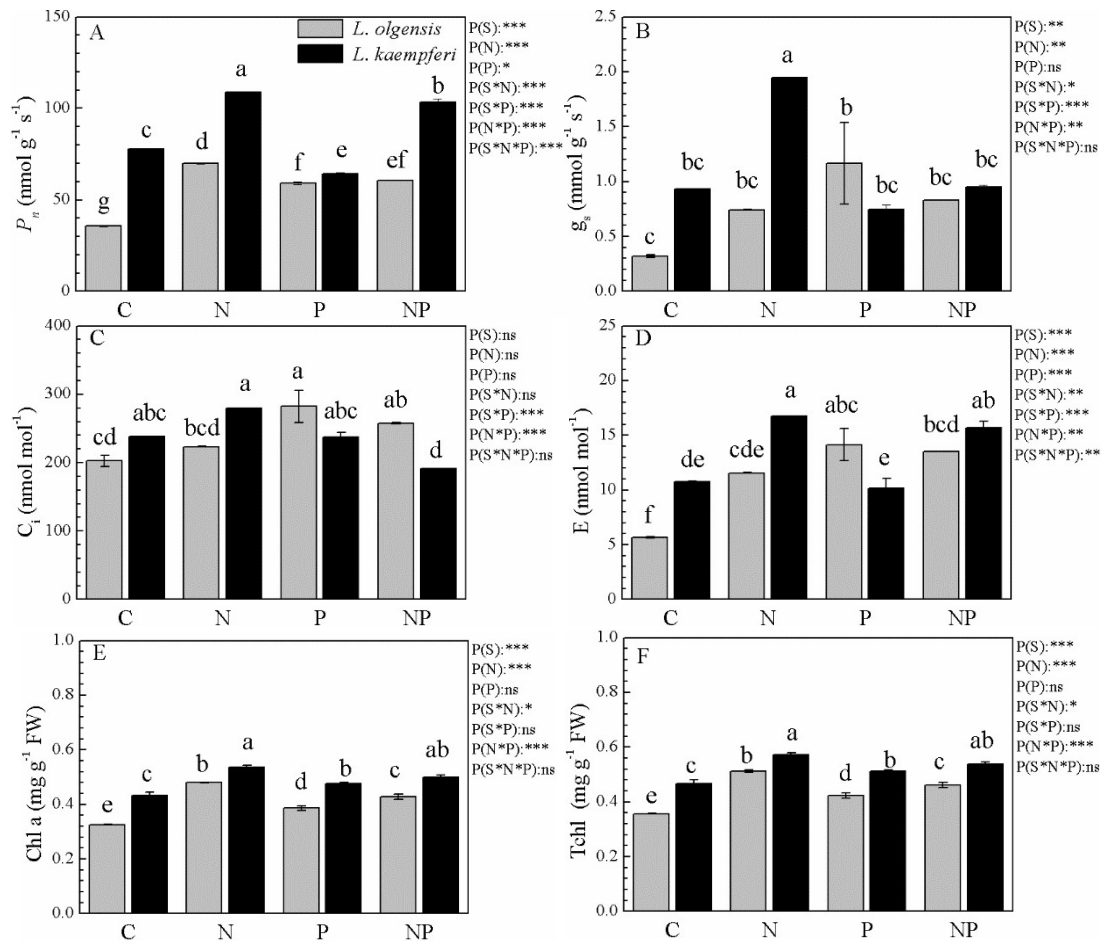
1 **Figure 1**



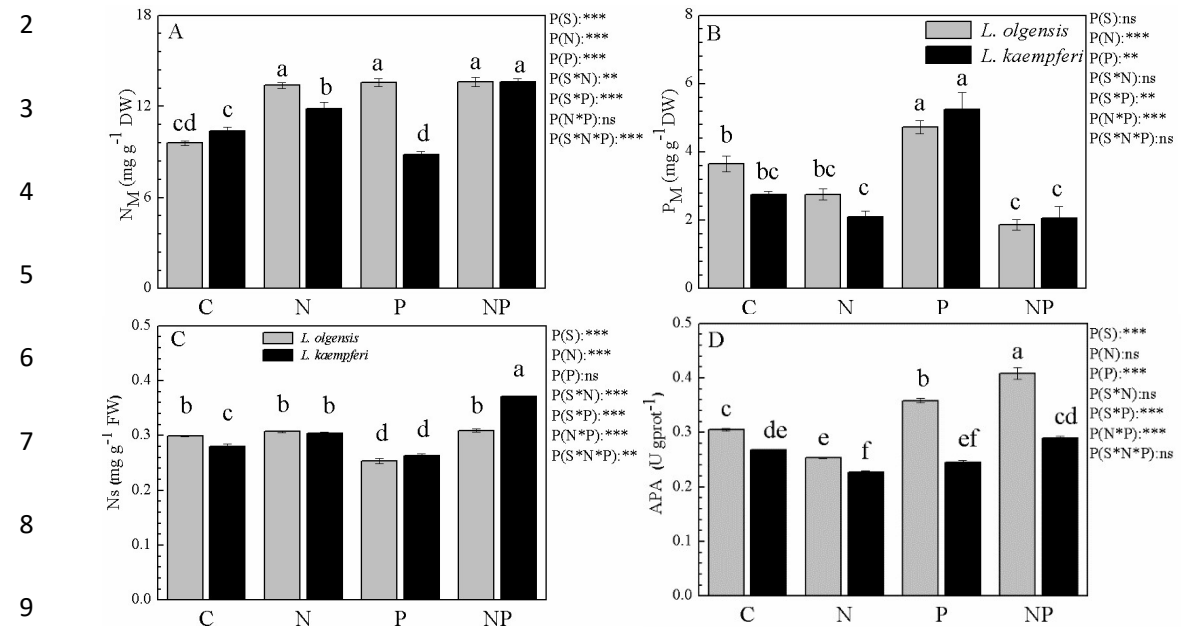
**Figure 2**



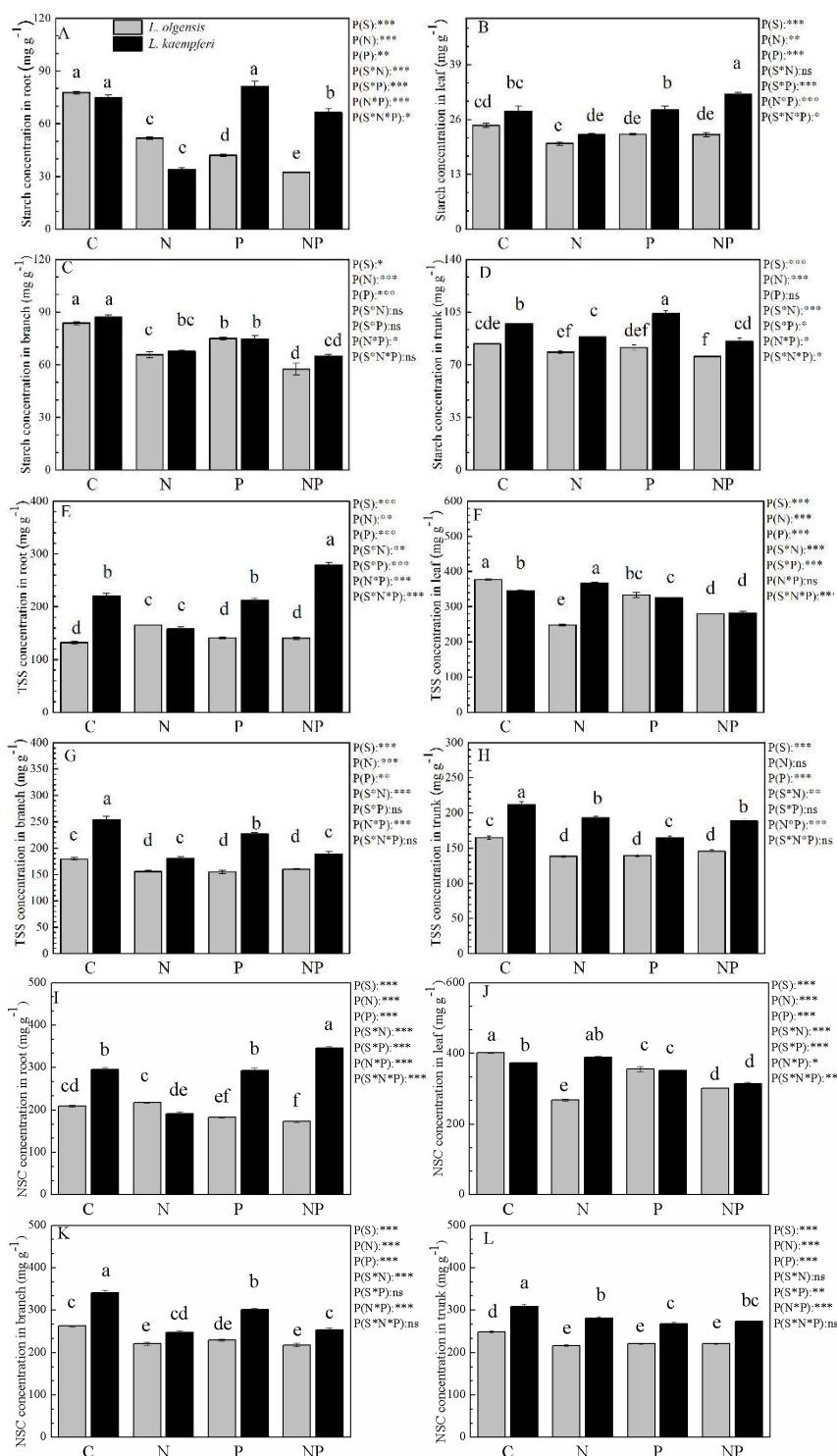
**Figure 3**



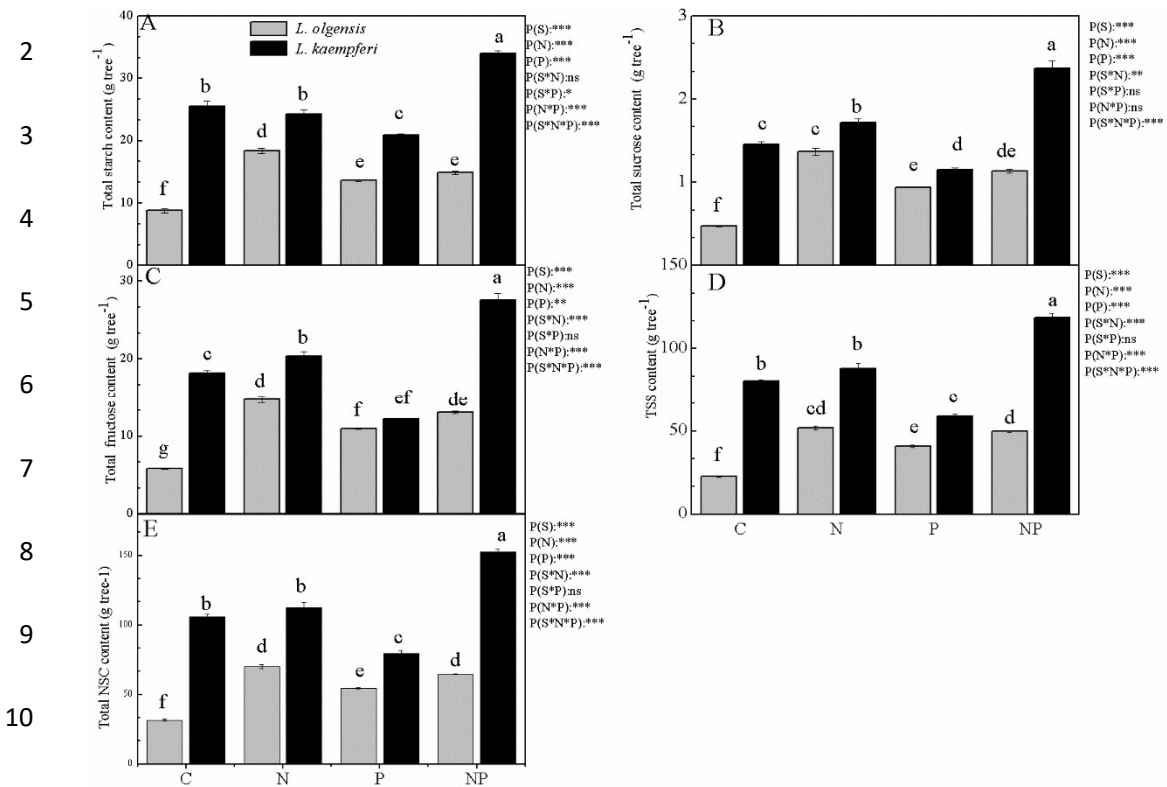
1 **Figure 4**



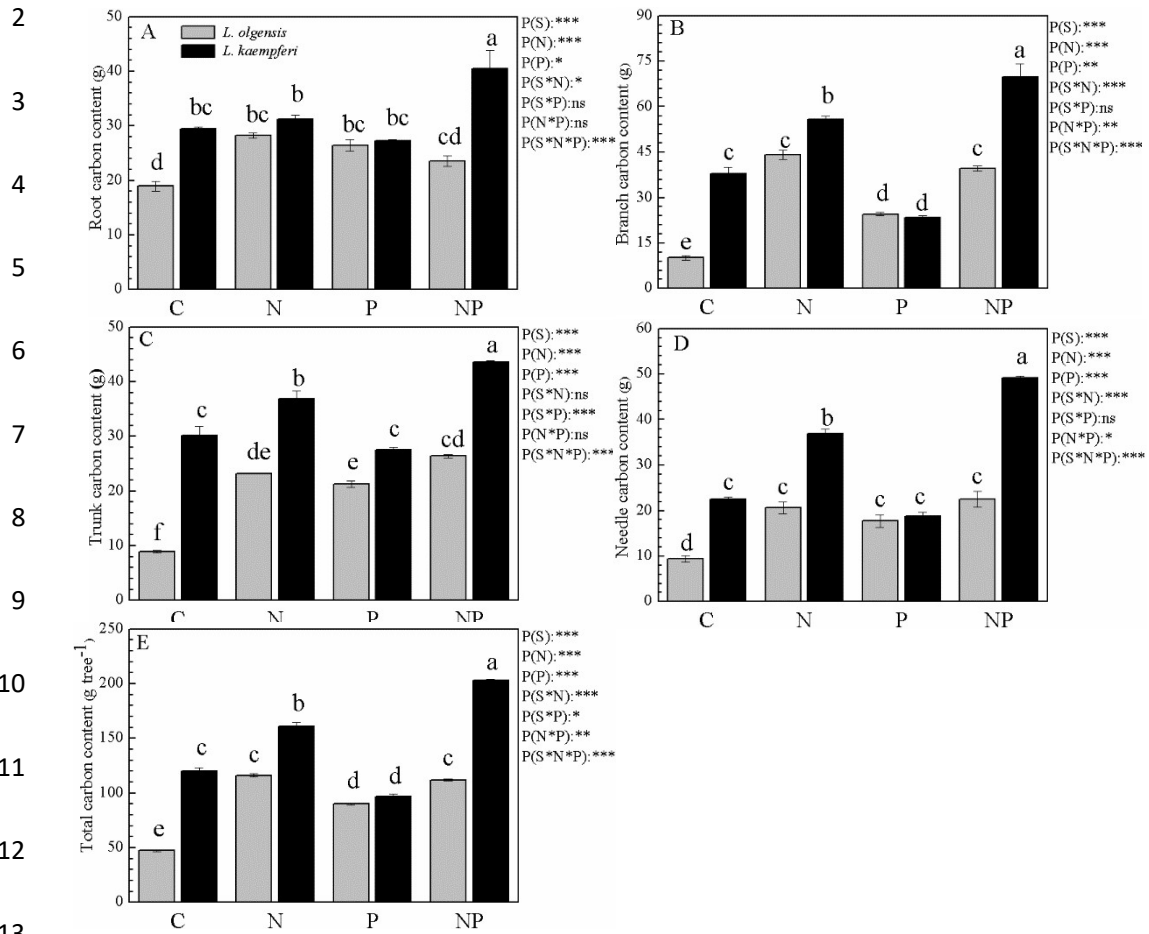
**Figure 5**



1 **Figure 6**



1 **Figure 7**





1 **Figure 8**

